Non-canonical inflammasome drives intrinsic anti-microbial responses in human Natural Killer cells

Nathan G.F. Leborgne[1,3], Jadie Acklam[1,3,5], Alexander J. Hogg[1,3,5],Zarema Albakova[1,3], Karen G. Hogg[1], Kim Samirah Robinson[1,2], Grant Calder[1], Arnel Villamin Jr[1,3], Callum Robson,[1,3], Freya Pealing[1,3] ,Matthew A. Care[1,3], Monika Gonka[1,3], James M. Fox[1], James Iremonger[4], James P. Hewitson[1,3,5], David G. Kent[1,3,5], Alison M. Layton[2], Frances E. Pearson[4], Max E. Noble[4], William Grey[1,3], Jillian L. Barlow[1,3,6], Dave Boucher[1,3,6,7]

1 York Biomedical Research Institute, University of York, Biology Department, Wentworth Way, York, YO10 5DD
2 Hull York Medical School, University of York, York, United Kingdom
3 Center for Blood Research
4 VISFO, The Old Flour Mill, Mill Royd Street, Brighouse, West Yorkshire, HD6 1EY
5 These authors contributed equally
6 Senior Author
7 Lead contact: Correspondence to dave.boucher@york.ac.uk

Short Title: A specialised inflammasome in human Natural Killer (NK) cells

Characters (excluding spaces): 19,074

Abstract

Inflammasomes are critical in rapidly responding to infections and tissue damage, with current paradigms centred on macrophages and epithelial cells. Natural Killer (NK) cells are innate lymphoid cells essential for infection and sepsis, and are an important focus for cell-based immunotherapy. Inflammasome-derived cytokines control their activity however, potential intrinsic inflammasome functions remain unknown in these cells. Here, we use *Salmonella enterica* Typhimurium to show that human NK cells sense infection via the non-canonical inflammasome (NCI), triggering pyroptosis and interleukin (IL)-18 secretion, without canonical inflammasome activation. Interestingly, the NK non-canonical inflammasome can be primed by IL-12 but not interferon-γ, distinguishing it from the interferon-primed NCI in other cell types. In addition, although *Salmonella* infection in NK cells reduces over time, we identify persistent infection in a proportion of these cells. Furthermore, we see an inflammasome-dependent enhanced responsiveness to reinfection, suggesting a long-term impact of the inflammasome on NK cells. Overall, the NCI is specialised and distinct in human NK cells.

159 words

Introduction

Innate immune responses are controlled by signaling pathways that enable coordination of immunity for rapid elimination of pathogens (1). Inflammasomes are critical signalling platforms controlling these responses to sterile and infectious agents in immune and epithelial cells (2,3). The inflammasome pathway senses triggers using pattern-recognition receptors (PRRs), including members of the Nod-like Receptor family (Nod-like receptor protein-1, NLRP1, and -3, NLRP3) and the NLR family CARD domain-containing protein 4 (NLRC4) (3). Unlike the canonical inflammasome, which activates caspase-1 upon detection of bacterial ligands (e.g., flagellin, nucleic acids), the non-canonical inflammasome (NCI) activates caspase-4 and caspase-5 (human) and caspase-11 (mice) upon recognition of cytosolic bacterial lipopolysaccharides (LPS) (4–6). In non-phagocytic cells, such as epithelial cells, LPS is presented to caspase-4 through interferon-inducible proteins called guanylate-binding proteins (GBPs) (7–11). GBP1 in particular, is involved in NCI activation in human macrophages upon *Salmonella* infection. NCI activation leads to the maturation and secretion of pro-inflammatory cytokines (12) and to pyroptosis, a form of pro-inflammatory cell death (13). Pyroptosis is driven by the caspase-mediated cleavage of the pore-forming protein Gasdermin D (GSDMD)(13,14) and leads to a Ninjurin-1 (NINJ1)-dependent plasma membrane rupture (15). This enables the control of infections, limiting their spread and mobilising further immune cells for clearance (16).

Inflammasomes are understood to influence adaptive responses in B and T cells through cytokine release, and there is evidence of cell-intrinsic inflammasome-dependent triggering pyroptosis in naive T cells (17–19). As such, inflammasomes are an important part of strong immune responses to vaccines and long-term defence against pathogens (20,21). Specifically, inflammasome activation in macrophages and epithelial cells induces the maturation and secretion of interleukin-18 (IL-18) and IL-1β. These cytokines generate antimicrobial responses in myeloid, lymphoid, and epithelial cells (12). Indeed, it is well established that along with IL-12, IL-18 can activate NK cells and promote the expression of interferon-γ (IFNγ) and cytotoxic effectors (22). Thus, innate lymphoid cells (ILC), including NK cells, are currently viewed as a downstream effector in the inflammasome pathway (19). However, whether NK cells possess intrinsic inflammasome activity remains unexplored.

Discovered in the 1970s, NK cells form part of the now expanded family of ILCs, including ILC1s, ILC2s, ILC3s, and lymphoid tissue-inducer cells (23). They play key roles in viral and tumour responses and are also increasingly studied *ex vivo* as part of novel immunotherapy approaches against cancer (24). In humans, recent advances in sequencing technologies have expanded our understanding of NK cell heterogeneity based on gene and protein expression (25,26). However, functionally these cells are still broadly divided into cytotoxic NK cells (release of perforin, granzyme and defined as $CD56^{dim}CD16^{+}$) and immunoregulatory NK cells (IL-12- and IL-18-dependent release of proinflammatory cytokines, IFNγ, CCL3, GM-CSF and defined as $CD56^{high}CD16^{low}$) (27,28). Thus, our understanding of functional heterogeneity in NK cells, particularly in response to infection, remains limited (29–31). Here, we investigate inflammasome functions in human NK cells following infection with the Gram-negative bacteria *Salmonella enterica* Typhimurium.

Results and Discussion

Human NK cells possess a functional non-canonical inflammasome

To date, inflammasome functions have mainly been studied in myeloid and epithelial cells where Gram-negative bacteria, such as *Salmonella*, are detected through exposure to a range of Pathogens-Associated Molecular Patterns (PAMPs), including the type III secretion system, flagellin and LPS (32,33). Much progress has been made in recent years on the contribution of innate lymphoid cells to early responses during infection (34,35), however whether they can directly activate inflammasome following the recognition of PAMPs remains unexplored.

We took advantage of a substantial dataset of healthy human blood NK cells to profile the expression of inflammasome pathway components in these cells where gene and protein expression data in human blood NK cells from 13 healthy donors (36,270 high-quality filtered cells) were grouped functionally as, NK1 ($CD56^{dim}$ $CD16^{high}CD3^{-}CD127^{-}CD57^{-}CD2^{-}$, further divided as subtype A, B, and C), NK2 ($CD56^{high}CD16^{-}CD3^{-}CD127^{+}CD57^{-}$ $CD2^{+}$), NK3 ($CD56^{dim}CD16^{high}CD3^{-}CD127^{-}$ $CD57^{+}CD2^{-}$), and NKint (25). As expected, *GZMB, GZMA,* and *PRF1* had a high comparative mean expression in NK1 and NK3 cells (**Figure 1A**). Additionally, canonical inflammasome components (*NLRP3*, *NLRC4*), the pro-inflammatory cytokine IL-1β, and the receptor detecting extracellular LPS (*TLR4*), were not expressed (32,36,37). *CASP1* and *GSDMD* were detected in NK1 and NK3, but not in NK2s. However, we were able to observe increased mean expression of NCI components, including *CASP4*, *GBP1*, and *GBP2*, in the NK1 and NK3 cell subtype, compared to NK2s. Other inflammasome-related genes were even further restricted, with *GBP4* expressed only in NK1B and NK2, and *PYCARD* in NK1B and NK1C (**Figure 1A**). To exclude the possibility that this was blood-specific, we looked at gene expression in human foetal thymic (tissue-resident) NK cells, confirming that *NLRP3*, *NLRC4*, and *TLR4* were not expressed. We were able to identify a $CASP4^{+}GBP2^{+}$ NK cell population here, where *CASP1, GSDMD*, and *PRF1* were upregulated in cycling cells, as compared to non-cycling cells (**Supp. Figure 1A**). This suggests the ability of specific NK cell populations to respond to intracellular Gram-negative infections in different tissues.

To confirm the findings from this sequencing data we next isolated human peripheral blood NK cells and neutrophils, paired from the same donor and

performed quantitative PCR for inflammasome-related genes. Alongside expression of *GZMA*, NK cells also showed low gene expression of *CASP1*, *CASP4*, and *GSDMD*, with greater expression of *GBP2* detected (**Figure 1B**). This was compared to expression of these genes in neutrophils, but whilst they also showed gene expression of *TLR4* and *NLRP3,* NK cells did not (**Figure 1C and Supp. Figure 1B**). Analysis of human blood NK cells, using macrophages as a comparison, by western blot also indicated the protein expression of a lower, but detectable amount of caspase-1, caspase-4 and GBP1 in NK cells, and similar levels of GBP2 and GBP5 with NK unique proteoforms of GBP1 and GBP5 (**Figure 1D**). Similar proteoforms have been previously reported in HUVEC cells (38). Overall, these data suggest that human NK cells lack several canonical inflammasome elements, but possess NCI components at homeostasis.

To test the activation of the NCI in the context of infection, we next expanded negatively selected human peripheral blood NK (ExhNK) cells *in vitro* for 14 days, resulting in a highly enriched population containing all NK cell subpopulations, as compared to fresh blood (**Supp. Figure 1C and D**). We infected the ExhNK cells with *Salmonella enterica* Typhimurium, strain SL1344 and LT2, which has been shown to infect cell types such as macrophages and epithelial cells, activating canonical (NLRP3, NLRC4; caspase-1) and non-canonical (GBPs; caspase-4, -5) inflammasomes. Infection resulted in a statistically significant increase in pyroptotic cell death, as measured by LDH release (**Figure 2A**), compared to uninfected cells. This cell death was decreased with the addition of the caspase-1/-4 inhibitor, VX-765 (39) (**Figure 2A**) and upon infection with *SalΔorgA* (**Figure 2B**). Indeed, *Salmonella* enter the cytosol (8,9) by using a subset of effectors (40), including the needle injectisome protein, OrgA, where its deletion strongly reduces *Salmonella* invasion of non-phagocytic cells (32). Thus, our data not only suggest a caspase-1/-4-dependent cell death mechanism, but also that NK cell-specific pyroptosis can occur and likely requires intracellular *Salmonella* invasion.

Central to the activation of the NCI is the maturation and secretion of pro-inflammatory cytokines, such as IL-1□ and IL-18. Indeed, our data confirm that priming with LPS or IFNγ, or infection with *S.* Typhimurium induces IL-1□ gene expression (**Figure 2C**) and its secretion (**Figure 2D**) in human neutrophils and macrophages. However, IL-1□ is not produced to a detectable level by ExhNK cells in these same conditions from matched donors. Strikingly, *S.* Typhimurium infection

in ExhNK cells did induce a statistically significant production of IL-18, which was caspase-1/-4-dependent (**Figure 2E**).

As *Salmonella* has been shown to activate multiple inflammasomes during infection, we investigated the pathways involved in such a response. Interestingly, we could not detect the cleaved caspase-1 fragments (p20/p10) upon *Salmonella* infection (**Figure 2F**). Additionally, although treatment with Nigericin, a potassium ionophore that activates NLRP3, induced cell death, this was not NLRP3-dependent, as shown with the addition of its inhibitor, MCC950 (41) (**Supp. Figure 2A**). NLRP3-dependent caspase-1 cleavage was also not observed in ExhNK cells (**Supp. Figure 2B**). Moreover, Val-boroPro (VbP, NLRP1 inflammasome activator, (42)) did not lead to the cleavage of GSDMD in ExhNK cells as compared to a control keratinocyte cell line (N/TERT) treated with VbP (**Supp. Figure 2C - D**)

Our data suggests a distinct mechanism of *Salmonella* sensing in exhNK cells that does not involve canonical inflammasome signaling, as evidenced by the undetectable expression level of NLRC4 and NLRP3 and the absence of response to NLRP3 and NLRP1 triggers. Instead, hNK cells utilise a pathway that induces both inflammatory caspase-dependent pyroptosis, and IL-18 secretion, but not IL-1□. This is concurrent with expression of NCI components. In addition, the absence of *TLR4* expression in hNK cells, which has been shown to be important for the priming of the canonical inflammasome pathway, may explain the non-responsiveness of exhNK cells to NLRP3 triggers and the absence of IL-1β. This differs from the classical activation of these pathways in macrophages where NLRC4 and NLRP3 are known to contribute significantly to the innate detection of *Salmonella* (36,43). The inflammatory caspase-dependent secretion of IL-18 is similar to previous literature showing its cleavage by caspase-4 (8,9,14,44) and suggests that, like epithelial cells, ExhNK cells may use an autocrine mechanism to potentiate their activation during bacterial infection.

Salmonella Typhimurium can access the cytosol in human NK cells

Gram-negative bacteria can activate the NCI through a range of mechanisms, by directly invading the host cell cytoplasm or by secreting outer-membrane vesicles (5,45). Upon epithelial and myeloid cell infection, *Salmonella* localise and replicate in specialised vacuoles where they are protected from the immune system. However,

as established in gut epithelial cells, *Salmonella* can escape these vacuoles, allowing hyper-replication but also its detection by cytosolic sensors (46).

Therefore, we next sought to identify whether *Salmonella* could invade ExhNK cells by infecting them with *S.* Typhimurium LT2-TdTomato. Using flow cytometry, we showed that ~40% of cells were TdTomato$^+$ (**Figure 3A**), suggesting an efficient infection of ExhNK cells. This could not differentiate between intracellularly- and extracellularly-associated *Salmonella*, however. Fluorescence microscopy with fibronectin-adhered CD56$^+$ NK cells was therefore used, indicating the presence of Tdtomato$^+$ *S.* Typhimurium in both CD56$^+$ vacuole-like structures and in the cytosol (**Figure 3B**). To confirm that *Salmonella* could directly access the cytosol, we used *Salmonella* expressing a glucose-6-phosphate-dependent cytosolic eGFP-reporter (8). As glucose-6-phosphate is only present in the cytosol, only cytosolic *Salmonella* will express the fluorescent GFP reporter. A significant percentage of ExhNK cells (10 - 15%), were eGFP$^+$ (**Figure 3C**), similar to levels in epithelial cells (8), suggesting that *Salmonella* can invade the cytosol of a proportion of NK cells. Indeed, we observed a range of hNK cell-*Salmonella* interactions as shown in **Figure 3D**. This correlates with NCI activation, where we show by western blot the presence of the active caspase-4 p32 cleavage product (47) and the reduction in full-length GSDMD in ExhNK cells in response to *S.* Typhimurium (**Figure 3E**). In comparison, infection with a Gram-positive pathogen, *Staphylococcus* aureus, which is known to activate the canonical inflammasome, did not induce caspase-4 cleavage or reduce full-length GSDMD protein.

To confirm inflammatory caspase activation, we used the caspase-activity probe FLICA660 and showed that ~5% of total ExhNK cells were FLICA$^+$ upon *Salmonella* infection, compared to ~1% in uninfected cells, suggesting potential caspase-4 activation (**Figure 3F**). In alignment with our previous microscopy, CellView^{TM} analysis, which allows imaging generated using flow cytometry, indicates a range activation and infection states, with the existence of FLICA$^+$*Salmonella*-TdTomato$^-$, FLICA$^-$*Salmonella*-TdTomato$^+$, and FLICA$^+$*Salmonella*-TdTomato$^+$ NK cell populations (**Supp. Figure 2E**). Thus, a significant proportion of hNK cells can be intracellularly infected with *Salmonella*, and in some cells the bacteria have the ability to access the cytosol and activate the inflammasome (FLICA$^+$). To test the potential of hNK cells to act as an infectious reservoir for *Salmonella,* we tracked *S.* Typhimurium LT2-TdTomato infection over 14

days. At shorter timepoints (after 5 hrs, Day 0, and 24 hrs later, Day 1) initial infection occurs in a significant subset of ExhNK cells, ~25 - 32%. At day 1 to day 14 post-infection, this reduces from ~20% to ~10% (**Figure 3G**), identifying a small NK population as TdTomato^{+}, where *Salmonella* may persist in NK cell vacuoles.

Thus, our study is suggestive of a heterogeneity of infective states in hNK cells, where persistence, potentially in the vacuole, and access to cytosolic niches are both possible. Further study will be required to demonstrate whether these differences in persistence and access are related to particular hNK cell subsets. Whilst these findings are novel in our understanding of NK cells, *Salmonella* vacuolar persistence and entry into the cytosol have both previously been shown as important in macrophages and epithelial cells, respectively. In macrophages pathogenic persistence induces cellular reprogramming and enables antibiotic evasion (48), whereas in epithelial cells cytosolic access allows the hyperproliferation of *Salmonella* (8). Thus, all these mechanisms converge on the ability of *Salmonella* to create an intracellular niche that allows their survival in different cell types.

Mouse NK cells do not activate the non-canonical inflammasome

We next asked whether murine NK cells displayed similar features using published RNA-seq datasets (49), where gene expression changes were measured in innate-like lymphoid populations, as compared to macrophages, following LPS priming. Our analyses show that, in contrast to humans, mouse NK cells express *Tlr4*, as well as other non-canonical inflammasome-associated genes, such as *Gbp2*, *Casp4*, and *Gsdmd*, similar to hNK cells and other mouse innate lymphoid populations (**Supp. Figure 3A**) (50). These results were supported by qPCR analysis of murine wild type splenic NK cells (**Supp. Figure 3B**). In naive NK cells, we detected transcripts for *Casp1*, *Casp4*, *Gbp2*, *Gsdmd*, and *Ninj1*, which was comparable to expression seen in macrophages. Macrophages were additionally found to express transcripts for *Il18*, *Il1b*, and *Il1a* (also upregulated with LPS priming), however they were absent in NK cells (**Supp. Figure 3C**). Furthermore, we did not detect protein expression of full length caspase-11 (murine orthologue of human caspase-4) or Gbp2 (mouse orthologue of human GBP1) following LPS priming (**Supp. Figure 3D**). Of note, we did detect an inactive caspase-11 isoform at 13 kDa, reported in the Uniprot database to be a variant containing only the caspase-11 CARD domain. In contrast to hNK cells and despite expressing

inflammasome components at the gene level, murine NK cells do not express a functional caspase-11 and do not activate the NCI in response to LPS priming.

Inversely, hNK cells do not express TLR4, but instead use the non-canonical inflammasome to enable detection of intracellular bacterial infection. These data highlight a critical difference between human and murine inflammasomes at the cellular level. Mice have been extensively used to study NK cells and *Salmonella*-based immunotherapies (51,52). These studies highlighted an important role for NK-derived IFNγ in reducing metastasis in a *Salmonella* dependent-manner. However, as murine cells respond differently to *Salmonella* infection, revisiting the anti-metastatic impact of *Salmonella* on human NK cells will be crucial.

The non-canonical inflammasome in human NK cells is specialised and primed by IL-12

IFNγ priming in non-phagocytic and phagocytic cells has previously been identified to impact NCI activation (8–10). To investigate the effect of IFNγ on hNK cells, we primed resting ExhNK cells *in vitro* for 16 hrs with recombinant human (rh) IFNγ or with rhIL-12 . IL-12 has the ability to prime and enhance NK cell functions (53) but has not previously been shown to promote the NCI activation. The cytokine is also important for the human response to *Salmonella* as individuals with a deficiency in IL-12 signaling are especially sensitive to *Salmonella* infection (54–56).

Surprisingly, IFNγ did not induce expression of any NCI-related genes (*CASP4*, *CASP1*, *GBP1*, *GBP2*, *GBP5*, *GSDMD*, and *NINJ1*), as compared to unstimulated cells (**Figure 4A - C**). In contrast, IL-12 stimulation resulted in statistically significant increases in *GBP1* and *GSDMD*. Although this did not increase ExhNK cell death, as measured by LDH assay at 5 hrs post infection, immunoblotting did show an increase in caspase-4 and GSDMD protein cleavage. Ninj1 levels remained unchanged (**Figure 4D and E**). IL-12 priming also significantly increased IFNγ and CCL3 secretion by ExhNK cells, but was not enhanced further by *S.* Typhimurium infection (**Figure 4F**). Strikingly, the *Salmonella*-dependent IL-18 secretion we previously observed (**Figure 2E**) was increased upon IL-12, but not IFNγ priming, suggesting a potentiation of NCI activity and its specialisation in hNK cells (**Figure 4H**).

Finally, we wanted to understand the long-term impact of *Salmonella* infections on the NK cell population and further susceptibility to reinfection. Human peripheral blood NK cells were isolated (**Supp. Figure 1C and D**) and immediately infected with *S.* Typhimurium, with or without the caspase inhibitor VX-765. Uninfected cells were used as a control. Cells were cultured for 14 days in the presence of gentamicin and then reinfected again for 5 hrs, before being assayed for NCI activation. Analysis of cell death showed an increased response, up to 90%, as compared to cells that were not pre-infected (~60% cell death) (**Figure 4I**). The reinfection-driven increase in cell death was completely abrogated in cells that were treated with VX-765 during the primary infection. This suggests that primary exposure to *Salmonella* in ExhNK cells is able to affect secondary outcomes in a caspase 1/4-dependent manner that is important for cell survival, raising the possibility that memory-like NK cell phenotypes are induced as a consequence of repeated infection. Such a phenotype has been investigated in response to tumours and virally-infected cells (31) and can be triggered by antigen-dependent and independent (e.g., cytokines) mechanisms (57,58).

Although impacted by a range of infections and inflammatory conditions (19), the cell-intrinsic innate immune pathways in NK cells have so far been poorly defined. This report is, to our knowledge, the first evidence that hNK cells possess intrinsic anti-bacterial response via the NCI. Prior to this, ILC3s were the only innate lymphoid cell shown to activate the inflammasome in murine models of *Salmonella* infection (59). Activation was found to be caspase-1-dependent via the recognition of flagellin. This canonical inflammasome signaling reduced ILC3 number and IL-22 production, destroyed replicative niches, and was necessary for the control of disease severity. Although these researchers did not explore human ILC3 inflammasomes, critically, our report now suggests potential differences between the inflammasome network in mice and humans, given that hNK cells do not express the machinery to sense intracellular flagellin (*NLRC4*), but use the NCI to detect LPS. It will be important to explore these differences further in the future, given that our work shows overlap in downstream effects with murine ILC3s, including pyroptosis, which is likely to reduce bacterial proliferation and promote the recruitment of additional immune cells to help clear bacterial infection.

Our key finding that *Salmonella* induces IL-18 secretion in NK cells suggests the potential for autocrine signalling, which may perhaps be enhanced by IL-12 in the

microenvironment, adding to the network of innate cells that have previously been identified as IL-18-producers (epithelial and myeloid cells). Interestingly, it has been shown that NK cells subsets may differentially express the IL-18 receptor, for example, with recent work suggesting that NK2 cells have the highest expression (25). Therefore, IL-18 secretion induced by *Salmonella* could influence locally the types of NK cells locally in the tissue following infection.

In conclusion, NCI activation in hNK cells is likely to impact the immune microenvironment and tissues by potentially releasing alarmins, cytokines and cytotoxic molecules. For example, we hypothesise that the effects we see on pyroptosis and on the NCI, are perhaps important in the decreased NK cell numbers and increased cell death reported in Gram-negative septicemia patients, and could contribute to understanding patient prognosis (60). This highlights the importance of future work to understand the impact of the NK cell-specific NCI on bacterial infection, tissue damage, immune dysregulation, and in immunotherapy.

Material and Methods

Study approval

Work with human primary cells was performed under approval by the University of York Biology Ethic Committee (DB202111). Mice were bred and maintained in specific pathogen-free conditions in the University of York, Biological Services Facility. Surplus C57 BL/6 mouse tissue was used with the approval of the local AWERB committee, and under license with the UK Home Office.

Human immune cell isolation and expansion

Human NK cells, PBMCs and neutrophils were isolated from fresh blood in EDTA-coated tubes (BD Bioscience) or from blood cones (apheresis product, NHS) collected from anonymous healthy donors. Neutrophils (only from fresh blood) and NK cells were isolated from whole blood by negative selection using the EasySep Direct Neutrophil isolation kit (StemCell Technology, 19666) and EasySep Direct human NK isolation kit (StemCell Technology, 19665). To ensure a maximal NK purity, isolated NK cells were expanded for 14 days with Immunocult NK Cell Expansion Kit (StemCell Technology, 100-0711), according to manufacturer instructions. After 14 days, dead cells from NK culture were removed using EasySep Dead Cell Removal (Annexin V) Kit.

PBMCs were isolated from whole blood using Lymphocyte Separation Medium (Lonza, BESP1109E). Red blood cells were lysed using ACK lysis buffer (Gibco, A1049201) for 5 min at room temperature. PBMCs were differentiated into macrophages with 50 ng/ml rhM-CSF (Proteintech, HZ-1192) in RPMI, 10% FBS, 1% penicillin/streptomycin, and 1% glutamine for 7 days at 37°C, 5% CO_2.

Murine immune cell isolation and priming

C57BL/6J mouse spleens were collected in PBS with 2% FBS. Single cell suspensions were obtained by passing tissue through a 70µm cell strainer (Corning, 352350). Red blood cells were lysed using ammonium chloride solution for 5 minutes at room temperature. Neutrophils were isolated using an EasySep Direct Neutrophil isolation kit (StemCell Technology, 19855). NK cells (LiveCD45$^+$CD3CD4CD8CD19CD11bCD11cFcεR1Gr1 (lineage)$^-$NK1.1$^+$) and macrophages (LiveCD45$^+$CD3$^-$CD11b^{high}) were stained with flow cytometry antibodies (see **Supplementary Table 1**) and isolated by FACS using a MoFlo Astrios (Beckman Coulter, Summit software). Mouse NK cells and macrophages were primed with 100 ng/mL of LPS EK (Invivogen) for 3hrs or used directly for RNA extraction after isolation.

Bacterial infection, cell priming, and cell death analysis

ExhNK cells, neutrophils, and differentiated macrophages were plated in 12-well plates in Opti-MEM (GIBCO) at 1x10^6 cells/ml, 2.5x10^6 cells/m and 0.5x10^6 cells/ml, respectively. Cells were infected with either *Salmonella enterica* Serovar Typhimurium SL1344 or LT2, or *S. enterica*Δ*orgA*, LT2-TdTomato, LT2-pPuhpT-GFP, or *Staphylococcus aureus* grown in log phase. Briefly, bacterial strains were cultured in lysogeny broth (LB) medium overnight at 37°C on an orbital shaker. These were subcultured 1:40 and grown for 3h at 37°C to generate log phase cultures. Before infection, bacteria were collected by centrifugation, and washed twice with pre-warmed PBS. Cells were infected at MOI 50 for 1h at 37°C. After 1 hr, gentamicin was added at a final concentration of 100 µg/ml to kill extracellular bacteria and cells were incubated for 4 hrs at 37°C, 5% CO_2. As indicated, VX-765 (25 µM in DMSO, Sigma-Aldrich, 5.31372) was added 30 mins before inflammasome activation.

In some experiments, ExhNK, rested for 6 hours in NK medium without NK cell Expansion Supplement (StemCell Technology, 100-0711) and neutrophils were primed 3 hours with 100 ng/ml ultrapure LPS-EK (Invivogen, tlrl-peklps) or with 25 ng/ml rhIFNγ (Proteintech, HZ-1301) or 100 ng/ml rhIL-12 (Proteintech, HZ-1256) for 16 hours. NLRP3 inflammasome was activated using Nigericin (20 μM in EtOH, Sigma-Aldrich, N7143) for 3 hrs at 37°C. As indicated MCC950 (10 μM in H_2O, Sigma-Aldrich, PZ0280) were added to the cell culture media 30 min before inflammasome activation. NLRP1 was activated using 3 μM Val-boroPro (Vbp, 5.31465).

For cell death measurements lactate dehydrogenase release in the supernatants was measured using a CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, G1780). Absorbance was measured on a plate reader (Infinite M Plex, TECAN) at 490nm with a 650nm background subtraction following manufacturer's instructions.

Quantitative PCR analysis

mRNA from primary cells (mouse and human) were isolated using the Arcturus PicoPure Isolation kit (ThermoFisher, KIT0204) and cDNA was generated using the SuperScript IV First Strand kit (ThermoFischer, 18091050), using manufacturer's instructions. qPCR was performed using TaqMan probes (Life Technologies, **Supplementary Table 1**) on an AriaMx Real-time PCR system (Agilent).

Immunoblotting

Cell extracts were lysed with cell sample buffer (2% SDS, 66mM Tris pH7.6 reconstituted in NuPage 1X LDS buffer (Novex, P5878547) and run on 10% or 4 - 20% acrylamide gels (BioRad) with methanol-chloroform precipitated cell supernatant mixes (61). Gels were transferred on 45 μM nitrocellulose membrane using the TurboBlot transfer system (BioRad), and blocked in 5% skimmed milk TBST. Samples were then incubated overnight at room temperature in the primary antibody at 4°C and for 2 hrs the following day in the secondary antibody (**Supplementary Table 1**). Membrane was imaged using the Immobilon Forte ECL Substrate (VWR, WBLUF) using a ThermoFisher iBright 1500.

Flow cytometry

Single cell suspensions were incubated with anti-CD16/CD32 antibodies (Biolegend, Clone 93) followed by fluorochrome-conjugated antibodies as detailed in **Supplementary Table 1**. All samples were co-stained with a cell viability dye (Zombie YG581 or Aqua, Biolegend, 423123 and 423102). In some experiments with human blood NK cells after *Salmonella* infection, and following the addition of gentamicin, YVAD-FMK FLICA660 or FLICA-FAM was added following manufacturer instructions (1:1000, FLICA Caspase-1 kit, BIO-RAD, 9120 and ICT098) and cells were incubated for 1 hr at 37°C. Cells were washed twice with the 1x kit wash buffer. Untreated NK cells stained for FLICA were used as control. Sample acquisition used an LSR Fortessa, CytoFLEX LX (Beckman Coulter), or FACSDiscover™ S8 (BD Biosciences, CellView™ Image) and then visualised using FlowJo (version 10.10) software. For cell sorting, an Astrios (70 µm nozzle, Beckman Coulter, Summit software) was used.

Immunofluorescence microscopy

8-well chamber slides (Ibidi, 80821) were coated with 12.5 µg/ml fibronectin for 1 hr, washed with PBS and transferred to 4°C until use. NK cells were seeded directly into chamber slides at $4x10^5$ cells/well and immediately infected with log phase *Salmonella* LT2-TdTomato at MOI 50 for 1h at 37°C, 5% CO_2. Gentamicin was added directly to the culture medium and cells were cultured for 4 hrs at 37°C, 5% CO_2. Cells were subsequently fixed with 4% PFA (in PBS) for 10 min at room temperature and washed with PBS. Cells were blocked with 5% goat serum for 2 hrs, stained with rabbit anti-human NCAM (CD56, Cell Signalling, 99746) overnight, washed with PBS and stained with goat anti-rabbit IgG (H+L), AF488 conjugate (Cell Signalling, 4412) for 1 hr. Cells were washed with PBS and stained with DAPI (2µg/ml) (Biotum, 40043) ready for imaging on Zeiss Elyra 7.

Cytokine assays

Cytokine secretion in cell culture supernatants were assessed using standard sandwich ELISA , IL-1β (Invitrogen, 88-7261), CCL3 (Invitrogen, 88-7035) , IL-18 (R&D systems, DY318-05), GM-CSF (Invitrogen, 88-8337) , IL-22 (Invitrogen, 88-7522). according to the manufacturer's instructions. IFNγ concentration was measured using the Lumit IFNγ immunoassay (Promega, W6040) following the

manufacturer's instructions in a 384 well plate. Luminescence was measured on a plate reader (Infinite M Plex, TECAN).

Bioinformatical data analysis and statistics

Mouse RNA sequencing data was obtained from the GEO database (GSE143943, (49). The data was filtered for genes relevant to NCI and the mean expression values log transformed. Heat maps were generated in R Studio using the pheatmap package. Clustering was automatically applied to the gene list.

Human scRNAseq datasets were obtained from either the foetal immune cell atlas (https://developmental.cellatlas.io/#) or peripheral blood (https://storage.googleapis.com/haniffalab/meta-nk/PBMC_Noreg.h5ad) and mined to analyse tissue resident or blood NK cells, respectively. The published data was loaded with scanpy and the inflammasome component genes visualised in a violin or dotplot. The dotplot was grouped by the 6 NK clusters discovered in the original study, and the genes were standard-scaled across these clusters to visualize relative abundance of each gene across cell contexts.

Statistical analysis for most experiments was performed using Prism10 (GraphPad, San Diego, CA).

Acknowledgement

D.B. is supported by an MRC New Investigator Award (MR/Z504221/1), a Springboard Award (Academy of Medical Sciences and Wellcome Trust) and a Royal Society Grant. J.A. was supported by a GenerationResearch MSc Studentship and VISFO. J.L.B., D.G.K, and M.C. are supported by the NIHR Leeds Biomedical Research Centre (BRC) (NIHR203331), and J.L.B and D.G.K. by an MRC National Mouse Genetics Network grant (MC_PC_21043). D.G.K is additionally supported by a CRUK Programme Foundation Award (DCRPGF\100008), an MRC-AMED joint award (MR/V005502/1), and Blood Cancer UK (24014). M.G. was in receipt of a BBSRC White Rose DTP PhD Studentship (BB/T007222/1).
W.G. is supported by an MRC Career Development Fellowship (MR/X007146/1), Leukaemia U.K. (2021/JGF/002), an EHA bilateral collaborative award and the Children's Cancer and Leukaemia Group (CCLGA 2022 13 Grey). K.S.R is supported by the Academy of Medical Sciences Springboard (SBF009\1153), Royal

Society (RG\R1\241058) and Hull York Medical School Start-up. The authors thank the Centre for Blood Research (CBR), SUKIT and the InflammaZoom UK networks for helpful discussions and constructive criticism on this research. The authors thank the phlebotomist team at the University of York for their support with collecting fresh blood for experimental purposes and the donors who kindly provided the blood. N/TERT was a kind donation from Prof. James Rheinwald (62). We would like to thank Prof. Petr Broz, University of Lausanne, and Marjan van der Woude, University of York, for providing the bacterial strains used for this study.

Authors contribution

N.G.F.L, J.A, A.H, Z.A, K.S.R, M.G, C.R, A.V, J.L.B, M.A.C, F.P. and D.B performed experiments. N.G.F.L, Z.A, J.H, J.L.B and D.B designed experiments, D.B secured funding, supervised, designed and oversaw the project. N.G.F.L, J.A, A.H, K.S.R, K.H, G.C, J.L.B, D.B analysed data. D.B, N.G.F.L, and J.L.B wrote the paper with inputs from all authors.

References

1. Janeway CA. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol. 1989;54 Pt 1:1–13.

2. Schroder K, Tschopp J. The inflammasomes. Cell. 2010 Mar 19;140(6):821–32.

3. Fu J, Schroder K, Wu H. Mechanistic insights from inflammasome structures. Nat Rev Immunol. 2024 July;24(7):518–35.

4. Ross C, Chan AH, von Pein JB, Maddugoda MP, Boucher D, Schroder K. Inflammatory Caspases: Toward a Unified Model for Caspase Activation by Inflammasomes. Annu Rev Immunol. 2022 Apr 26;40:249–69.

5. Rathinam VAK, Zhao Y, Shao F. Innate immunity to intracellular LPS. Nat Immunol. 2019 May;20(5):527–33.

6. Grant S, Boucher D. 10 things you wish you knew about inflammatory caspases: a holistic review of their biochemistry, activation, and signalling. J Biol Chem. 2025 Aug 2;110557.

7. Tretina K, Park ES, Maminska A, MacMicking JD. Interferon-induced guanylate-binding proteins: Guardians of host defense in health and disease. J Exp Med. 2019 Mar 4;216(3):482–500.

8. Santos JC, Boucher D, Schneider LK, Demarco B, Dilucca M, Shkarina K, et al. Human GBP1 binds LPS to initiate assembly of a caspase-4 activating platform on cytosolic bacteria. Nat Commun. 2020 June 24;11(1):3276.

9. Wandel MP, Kim BH, Park ES, Boyle KB, Nayak K, Lagrange B, et al. Guanylate-binding proteins convert cytosolic bacteria into caspase-4 signaling platforms. Nat Immunol. 2020 Aug;21(8):880–91.

10. Fisch D, Bando H, Clough B, Hornung V, Yamamoto M, Shenoy AR, et al. Human GBP1 is a microbe-specific gatekeeper of macrophage apoptosis and pyroptosis. EMBO J. 2019 July 1;38(13):e100926.

11. Kutsch M, Sistemich L, Lesser CF, Goldberg MB, Herrmann C, Coers J. Direct binding of polymeric GBP1 to LPS disrupts bacterial cell envelope functions. EMBO J. 2020 July 1;39(13):e104926.

12. Chan AH, Schroder K. Inflammasome signaling and regulation of interleukin-1 family cytokines. J Exp Med. 2020 Jan 6;217(1):e20190314.

13. Broz P, Pelegrín P, Shao F. The gasdermins, a protein family executing cell death and inflammation. Nat Rev Immunol. 2020 Mar;20(3):143–57.

14. Wang K, Sun Q, Zhong X, Zeng M, Zeng H, Shi X, et al. Structural Mechanism for GSDMD Targeting by Autoprocessed Caspases in Pyroptosis. Cell. 2020 Mar 5;180(5):941-955.e20.

15. Kayagaki N, Kornfeld OS, Lee BL, Stowe IB, O'Rourke K, Li Q, et al. NINJ1 mediates plasma membrane rupture during lytic cell death. Nature. 2021 Mar;591(7848):131–6.

16. Chen KW, Broz P. Gasdermins as evolutionarily conserved executors of inflammation and cell death. Nat Cell Biol. 2024 Sept;26(9):1394–406.

17. Linder A, Hornung V. Inflammasomes in T cells. J Mol Biol. 2022 Feb 28;434(4):167275.

18. Linder A, Bauernfried S, Cheng Y, Albanese M, Jung C, Keppler OT, et al. CARD8 inflammasome activation triggers pyroptosis in human T cells. EMBO J. 2020 Oct 1;39(19):e105071.

19. Deets KA, Vance RE. Inflammasomes and adaptive immune responses. Nat Immunol. 2021 Apr;22(4):412–22.

20. Reinke S, Pantazi E, Chappell GR, Sanchez-Martinez A, Guyon R, Fergusson JR, et al. Emulsion and liposome-based adjuvanted R21 vaccine formulations mediate protection against malaria through distinct immune mechanisms. Cell Rep Med. 2023 Nov 21;4(11):101245.

21. Muñoz-Wolf N, Ward RW, Hearnden CH, Sharp FA, Geoghegan J, O'Grady K, et al. Non-canonical inflammasome activation mediates the adjuvanticity of nanoparticles. Cell Rep Med. 2023 Jan;4(1):100899.

22. Munder M, Mallo M, Eichmann K, Modolell M. Murine macrophages secrete interferon gamma upon combined stimulation with interleukin (IL)-12 and IL-18: A novel pathway of autocrine macrophage activation. J Exp Med. 1998 June 15;187(12):2103–8.

23. Vivier E, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate Lymphoid Cells: 10 Years On. Cell. 2018 Aug 23;174(5):1054–66.

24. Tang F, Li J, Qi L, Liu D, Bo Y, Qin S, et al. A pan-cancer single-cell panorama of human natural killer cells. Cell. 2023 Sept;186(19):4235-4251.e20.

25. Rebuffet L, Melsen JE, Escalière B, Basurto-Lozada D, Bhandoola A, Björkström NK, et al. High-dimensional single-cell analysis of human natural killer cell heterogeneity. Nat Immunol. 2024 Aug;25(8):1474–88.

26. Crinier A, Milpied P, Escalière B, Piperoglou C, Galluso J, Balsamo A, et al. High-Dimensional Single-Cell Analysis Identifies Organ-Specific Signatures and Conserved NK Cell Subsets in Humans and Mice. Immunity. 2018 Nov 20;49(5):971-986.e5.

27. Lanier LL, Le AM, Civin CI, Loken MR, Phillips JH. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. J Immunol Baltim Md 1950. 1986 June 15;136(12):4480–6.

28. Nagler A, Lanier LL, Cwirla S, Phillips JH. Comparative studies of human FcRIII-positive and negative natural killer cells. J Immunol. 1989 Nov 15;143(10):3183–91.

29. Mody CH, Ogbomo H, Xiang RF, Kyei SK, Feehan D, Islam A, et al. Microbial killing by NK cells. J Leukoc Biol. 2019 June;105(6):1285–96.

30. Schmidt S, Zimmermann SY, Tramsen L, Koehl U, Lehrnbecher T. Natural Killer Cells and Antifungal Host Response. Clin Vaccine Immunol. 2013 Apr;20(4):452–8.

31. Björkström NK, Strunz B, Ljunggren HG. Natural killer cells in antiviral immunity. Nat Rev Immunol. 2022 Feb;22(2):112–23.

32. Bierschenk D, Boucher D, Schroder K. Salmonella-induced inflammasome activation in humans. Mol Immunol. 2017 June;86:38–43.

33. Clare B. Inflammasome activation by Salmonella. Curr Opin Microbiol. 2021 Dec;64:27–32.

34. Ryu S, Lim M, Kim J, Kim HY. Versatile roles of innate lymphoid cells at the mucosal barrier: from homeostasis to pathological inflammation. Exp Mol Med. 2023 Sept 11;55(9):1845–57.

35. Eken A, Johnson S, Erdem S, Hsieh EWY. Phantom of the immunologic opera: Unmasking the role of innate lymphoid cells (ILC) in inborn errors of immunity (IEI). J Hum Immun. 2025 Nov 3;1(4):e20250045.

36. Gram AM, Wright JA, Pickering RJ, Lam NL, Booty LM, Webster SJ, et al. Salmonella Flagellin Activates NAIP/NLRC4 and Canonical NLRP3 Inflammasomes in Human Macrophages. J Immunol Baltim Md 1950. 2021 Feb 1;206(3):631–40.

37. Naseer N, Egan MS, Reyes Ruiz VM, Scott WP, Hunter EN, Demissie T, et al. Human NAIP/NLRC4 and NLRP3 inflammasomes detect Salmonella type III secretion system activities to restrict intracellular bacterial replication. PLoS Pathog. 2022 Jan;18(1):e1009718.

38. Naschberger E, Geißdörfer W, Bogdan C, Tripal P, Kremmer E, Stürzl M, et al. Processing and secretion of guanylate binding protein‐1 depend on inflammatory caspase activity. J Cell Mol Med. 2017 Sept;21(9):1954–66.

39. Wannamaker W, Davies R, Namchuk M, Pollard J, Ford P, Ku G, et al. (S)-1-((S)-2-{[1-(4-Amino-3-chloro-phenyl)-methanoyl]-amino}-3,3-dimethyl-butanoyl)-pyrrolidine-2-carboxylic acid ((2 R,3 S)-2-ethoxy-5-oxo-tetrahydro-furan-3-yl)-amide (VX-765), an Orally Available Selective Interleukin (IL)-Converting Enzyme/Caspase-1 Inhibitor, Exhibits Potent Anti-Inflammatory Activities by Inhibiting the Release of IL-1β and IL-18. J Pharmacol Exp Ther. 2007 May;321(2):509–16.

40. Agbor TA, McCormick BA. Salmonella effectors: important players modulating host cell function during infection. Cell Microbiol. 2011 Dec;13(12):1858–69.

41. Coll RC, Hill JR, Day CJ, Zamoshnikova A, Boucher D, Massey NL, et al. MCC950 directly targets the NLRP3 ATP-hydrolysis motif for inflammasome inhibition. Nat Chem Biol. 2019 June;15(6):556–9.

42. Okondo MC, Johnson DC, Sridharan R, Go EB, Chui AJ, Wang MS, et al. DPP8 and DPP9 inhibition induces pro-caspase-1-dependent monocyte and macrophage pyroptosis. Nat Chem Biol. 2017 Jan;13(1):46–53.

43. Man SM, Hopkins LJ, Nugent E, Cox S, Glück IM, Tourlomousis P, et al. Inflammasome activation causes dual recruitment of NLRC4 and NLRP3 to the same macromolecular complex. Proc Natl Acad Sci U S A. 2014 May 20;111(20):7403–8.

44. Devant P, Dong Y, Mintseris J, Ma W, Gygi SP, Wu H, et al. Structural insights into cytokine cleavage by inflammatory caspase-4. Nature. 2023 Dec;624(7991):451–9.

45. Vanaja SK, Russo AJ, Behl B, Banerjee I, Yankova M, Deshmukh SD, et al. Bacterial Outer Membrane Vesicles Mediate Cytosolic Localization of LPS and Caspase-11 Activation. Cell. 2016 May;165(5):1106–19.

46. Chong A, Cooper KG, Kari L, Nilsson OR, Hillman C, Fleming BA, et al. Cytosolic replication in epithelial cells fuels intestinal expansion and chronic fecal shedding of Salmonella Typhimurium. Cell Host Microbe. 2021 July;29(7):1177-1185.e6.

47. Chan AH, Burgener SS, Vezyrgiannis K, Wang X, Acklam J, Von Pein JB, et al. Caspase-4 dimerisation and D289 auto-processing elicit an interleukin-1β-converting enzyme. Life Sci Alliance. 2023 Oct;6(10):e202301908.

48. Stapels DAC, Hill PWS, Westermann AJ, Fisher RA, Thurston TL, Saliba AE, et al. *Salmonella* persisters undermine host immune defenses during antibiotic treatment. Science. 2018 Dec 7;362(6419):1156–60.

49. Kim EY, Ner-Gaon H, Varon J, Cullen AM, Guo J, Choi J, et al. Post-sepsis immunosuppression depends on NKT cell regulation of mTOR/IFN-γ in NK cells. J Clin Invest. 2020 June 1;130(6):3238–52.

50. Chen D, Zhao Y, Feng Y, Jin C, Yang Q, Qiu H, et al. Expression of TLR2, TLR3, TLR4, and TLR7 on pulmonary lymphocytes of *Schistosoma japonicum* -infected C57BL/6 mice. Innate Immun. 2019 May;25(4):224–34.

51. Yang S, Sheffer M, Kaplan IE, Wang Z, Tarannum M, Dinh K, et al. Non-pathogenic E. coli displaying decoy-resistant IL18 mutein boosts anti-tumor and CAR NK cell responses. Nat Biotechnol. 2024 Oct 4;

52. Lin Q, Rong L, Jia X, Li R, Yu B, Hu J, et al. IFN-γ-dependent NK cell activation is essential to metastasis suppression by engineered Salmonella. Nat Commun. 2021 May 5;12(1):2537.

53. Tait Wojno ED, Hunter CA, Stumhofer JS. The Immunobiology of the Interleukin-12 Family: Room for Discovery. Immunity. 2019 Apr;50(4):851–70.

54. Jong RD, Altare F, Haagen IA, Elferink DG, Boer TD, Van Breda Vriesman PJC, et al. Severe Mycobacterial and *Salmonella* Infections in Interleukin-12 Receptor-Deficient Patients. Science. 1998 May 29;280(5368):1435–8.

55. Staretz-Haham O, Melamed R, Lifshitz M, Porat N, Fieschi C, Casanova JL, et al. Interleukin-12 Receptor β1 Deficiency Presenting as Recurrent Salmonella Infection. Clin Infect Dis. 2003 July 1;37(1):137–40.

56. Ottenhoff THM, Verreck FAW, Lichtenauer-Kaligis EGR, Hoeve MA, Sanal O, van Dissel JT. Genetics, cytokines and human infectious disease: lessons from weakly pathogenic mycobacteria and salmonellae. Nat Genet. 2002 Sept;32(1):97–105.

57. Cerwenka A, Lanier LL. Natural killer cell memory in infection, inflammation and cancer. Nat Rev Immunol. 2016 Feb;16(2):112–23.

58. O'Sullivan TE, Sun JC, Lanier LL. Natural Killer Cell Memory. Immunity. 2015 Oct;43(4):634–45.

59. Xiong L, Wang S, Dean JW, Oliff KN, Jobin C, Curtiss R, et al. Group 3 innate lymphoid cell pyroptosis represents a host defence mechanism against Salmonella infection. Nat Microbiol. 2022 June 6;7(7):1087–99.

60. Andaluz-Ojeda D, Iglesias V, Bobillo F, Almansa R, Rico L, Gandía F, et al. Early natural killer cell counts in blood predict mortality in severe sepsis. Crit Care. 2011 Oct 21;15(5):R243.

61. Groß O. Measuring the Inflammasome. In: Ashman RB, editor. Leucocytes [Internet]. Totowa, NJ: Humana Press; 2012 [cited 2025 July 6]. p. 199–222. (Methods in Molecular Biology; vol. 844). Available from: https://link.springer.com/10.1007/978-1-61779-527-5_15

62. Dickson MA, Hahn WC, Ino Y, Ronfard V, Wu JY, Weinberg RA, et al. Human Keratinocytes That Express hTERT and Also Bypass a $p16^{INK4a}$ -Enforced Mechanism That Limits Life Span Become Immortal yet Retain Normal Growth and Differentiation Characteristics. Mol Cell Biol. 2000 Feb 1;20(4):1436–47.

Figure 1. Human blood NK cells express components of the non-canonical inflammasome. (**A**) scRNA-seq data of inflammasome components in subsets of human peripheral blood NK cells showing the relative abundance (standard-scaled) and percentage of cells expressing inflammasome components, (n=19). (**B**) Quantitative PCR analysis of non-canonical inflammasome components in unexpanded human peripheral blood NK cells, n = 4 donors (**C**) Quantitative PCR analysis of *TLR4* and *NLRP3* in unexpanded peripheral blood NK cells and neutrophils from four matched donors. (**D**) Western blot analysis of expanded human peripheral blood NK cells and macrophages from 2 matched donors compared to loading controls of αTubulin and H3. *[1] and *[2] indicate NK cell-specific protein isoforms of GBP1 and GBP5, respectively. Western blot is representative of 4 blood donors.

Figure 2. *Salmonella* Typhimurium infection induces cell death and IL-18 production in human blood NK cells. Expanded human peripheral blood NK cells were infected with *S.* Typhimurium or *S.* Typhimurium*ΔorgA*, and with or without VX-765. At 5 hrs post-infection, cells were assessed by (**A** and **B**) LDH assay for cell death, (**C**) western blot to analyse caspase-1 cleavage, and supernatant by ELISA for (**E**) IL-1beta and (**F**) IL-18 production, n = 6 - 9 donors across 2 - 3 independent experiments. (**D**) Quantitative PCR analysis of *IL1B* following priming with LPS (for 3 hrs) and IFNgamma (for 16 hrs). In (**D**) and (**E**) NK cells were compared to neutrophils and macrophages from matching donors.

Figure 3. *Salmonella* Typhimurium accesses the cytosol and persists in human blood NK cells. Expanded human peripheral blood NK cells were infected with *S.* Typhimurium-TdTomato. (**A**) At 5 hrs post-infection NK cells were stained on fibronectin coated slides with anti-CD56 and DAPI and visualised by fluorescence microscopy (white bar indicates 5 µm), or (**B**) were subjected to analysis by flow cytometry. (**C**) Alternatively, these NK cells were infected with *S.* Typhimurium, stably expressing a cytosolic eGFP reporter and analysed by flow cytometry. (**D**) Caspase-1 and -4 activation was measured by flow cytometry using the inflammatory caspase probe FLICA660 (**E**) Representative western blot analysis of expanded human peripheral blood NK cells infected with either *S.* Typhimurium or *Staphylococcus* aureus and with or without VX-765 for 5 hrs compared to loading

controls of H3. *[1] indicates a p32 cleavage product of active caspase-4. (**F**) Percentage of *S.* Typhimurium-TdTomato in NK cells was measured at days 1, 2, 5, 7, and 14 post-infection. Data from 2 independent experiments, n = 4 - 6 donors.

Figure 4. IL-12 but not IFNγ-mediated activation of human blood NK cells leads to upregulation of non-canonical inflammasome components. Expanded human peripheral blood NK cells were treated with and without rhIL-12 or rhIFNγ for 16 hrs. Quantitative PCR was used to assess expression of (**A**) *CASP4* and *CASP1*, (**B**) *GBP1* and *GBP2*, and (**C**) *GBP5*, *GSDMD*, and *NINJ1*. Post-treatment, NK cells were additionally infected with *S.* Typhimurium alone, or in combination with VX-765. Supernatant was taken at 5 hrs post-infection in order to assess (**D**) cell death by LDH release and (**F**) IFNγ, (**G**) CCL3, and (**H**) IL-18 production by ELISA. (**E**) Representative western blot analysis of expanded and primed human NK cells infected with either *S.* Typhimurium alone or in combination with VX-765 for 5 hrs compared to loading controls of H3. *[1] indicates a p32 cleavage product of caspase-4. *[2] indicates a p30 cleavage product of GSDMD. (**I**) NK cells were infected with *S.* Typhimurium, and expanded for 14 days prior to reinfection by *S.* Typhimurium and analysis of cell death by LDH release . Data from 2 - 3 independent experiments, n = 3 - 8 donors.

Supplementary Figure Legends

Supplementary Table 1. (**A**) Quantitative PCR Taqman primer sets for human and mouse targets. (**B**) Antibodies used in flow cytometry (FC), immunofluorescence (IF), and western blot (WB).

Supplementary Figure 1. Human thymic NK cells express components of the non-canonical inflammasome. (**A**) scRNA-seq data of inflammasome components in *CASP4*$^{+}$*GBP2*$^{+}$ and *CASP4*$^{-}$*GBP2*$^{-}$ human foetal thymic non-cycling and cycling NK cells showing the median expression and number of cells expressing inflammasome components. (**B**) Quantitative PCR analysis of non-canonical inflammasome components in neutrophils, n = 4 donors, matched with NK cell phenotypes in Fig. 1B. Human peripheral blood from (**C**) before and (**D**) after NK cell negative selection, analysed by flow cytometry to show typical percentages of CD56^{int}CD16^{+} and CD56^{high}CD16^{-}, and within this NK1, NK2, and NK3 subsets, as defined by Rebuffet *et al* (2024).

Supplementary Figure 2. NLRP1, NLRP3, and FLICA activation in human blood NK cells. Expanded human peripheral blood NK cells were treated with nigericin (20 µM), with or without MCC950 (10 µM) for 3 hrs. Cells were then assessed for (**A**) the release of LDH for cell death and (**B**) the cleavage of caspase-1 by western blot. (**C**) NK cells or (**D**) N/TERT keratinocytes were treated with Val-boroPro (3µM, VbP) for 6hrs, with and without VX-765, and assessed by western blot. n = 4 - 6 donors. (**E**) Representative images, using CellView™ beam-splitting technology, of CD56^{+} cells were reconstructed to show human NK cells that were FLICA-FAM^{+}*Salmonella*-TdTomato^{-} (NK cell 1), FLICA-FAM^{-}*Salmonella*-TdTomato^{+} (NK cell 2), and FLICA-FAM^{+}*Salmonella*-TdTomato^{+} (NK cell 3), respectively. *^{1} and *^{2} indicate possible NK cell-specific protein isoforms of CASPASE-1. *^{3} indicates the p3.0 cleavage fragment of GSDMD.

Supplementary Figure 3. Canonical and non-canonical inflammasome components are expressed in mouse NK cells at the gene, but not the protein level . (**A**) RNASeq dataset extracted from flow cytometry sorted immune cells showing a heatmap expression analysis of canonical and non-canonical

inflammasome-associated genes. Murine spleen NK cells and CD11b^{high} cells were sorted and cDNA was extracted from either (**B**) naive populations or (**C**) from LPS (100 ng/mL, 3 hrs)-primed cells. Expression of canonical and non-canonical inflammasome-associated genes was assessed by quantitative PCR using *Gapdh* as a house-keeping gene, n = 4 - 8, collected across 2 independent experiments. (**D**) Representative western blot analysis of caspase-11 and Gbp2 expression in LPS-primed murine spleen NK cells.

Figure 1

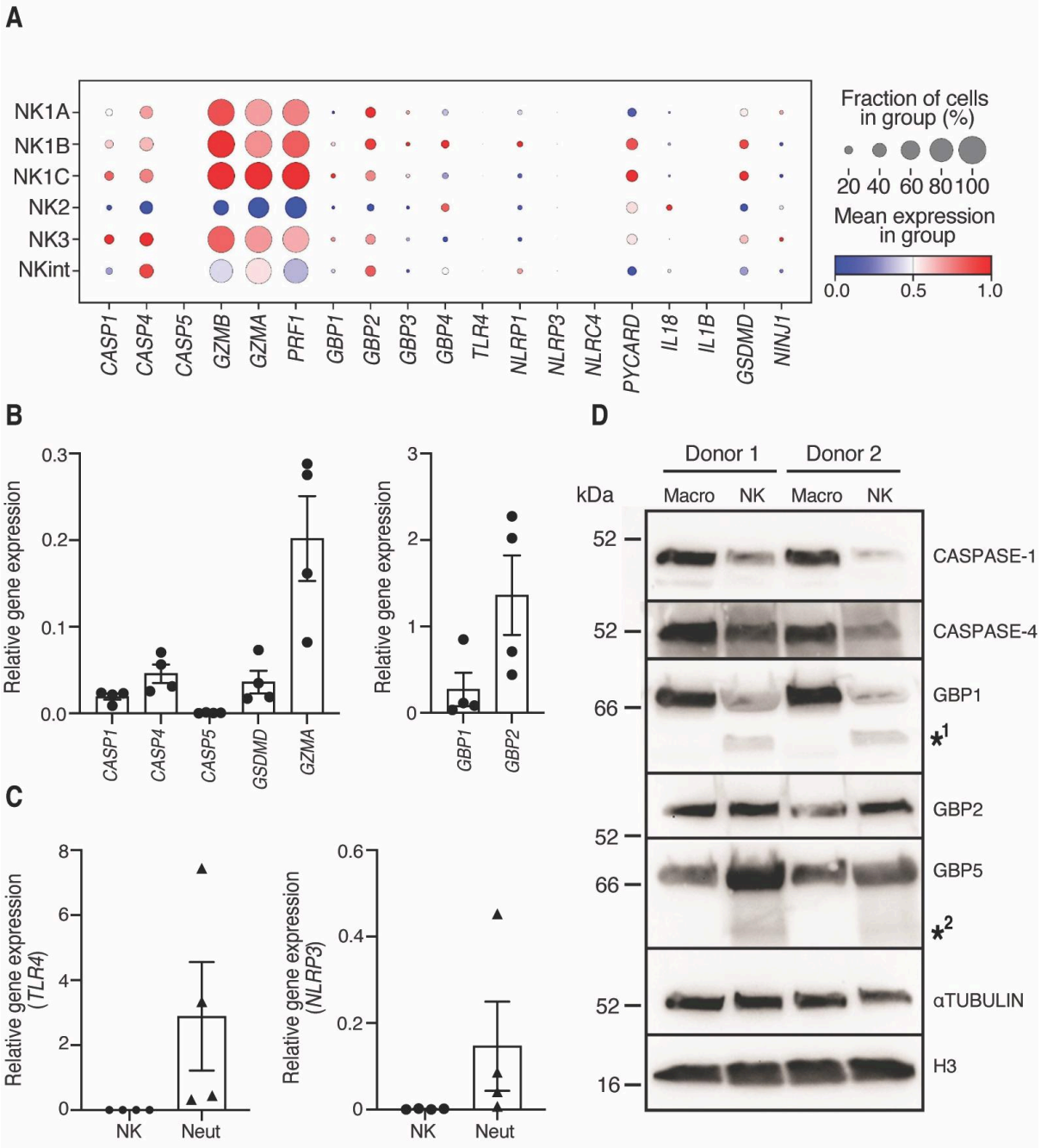

A
NK1A
NK1B
NK1C
NK2
NK3
NKint
CASP1
CASP4
CASP5
GZMB
GZMA
PRF1
GBP1
GBP2
GBP3
GBP4
TLR4
NLRP1
NLRP3
NLRC4
PYCARD
IL18
IL1B
GSDMD
NINJ1
Fraction of cells in group (%)
20 40 60 80 100
Mean expression in group
0.0 0.5 1.0
B
Relative gene expression
CASP1
CASP4
CASP5
GSDMD
GZMA
GBP1
GBP2
C
Relative gene expression (TLR4)
Relative gene expression (NLRP3)
NK
Neut
D
Donor 1
Donor 2
kDa
Macro
NK
CASPASE-1
CASPASE-4
GBP1
*1
GBP2
GBP5
*2
αTUBULIN
H3
52
66
16

Figure 2

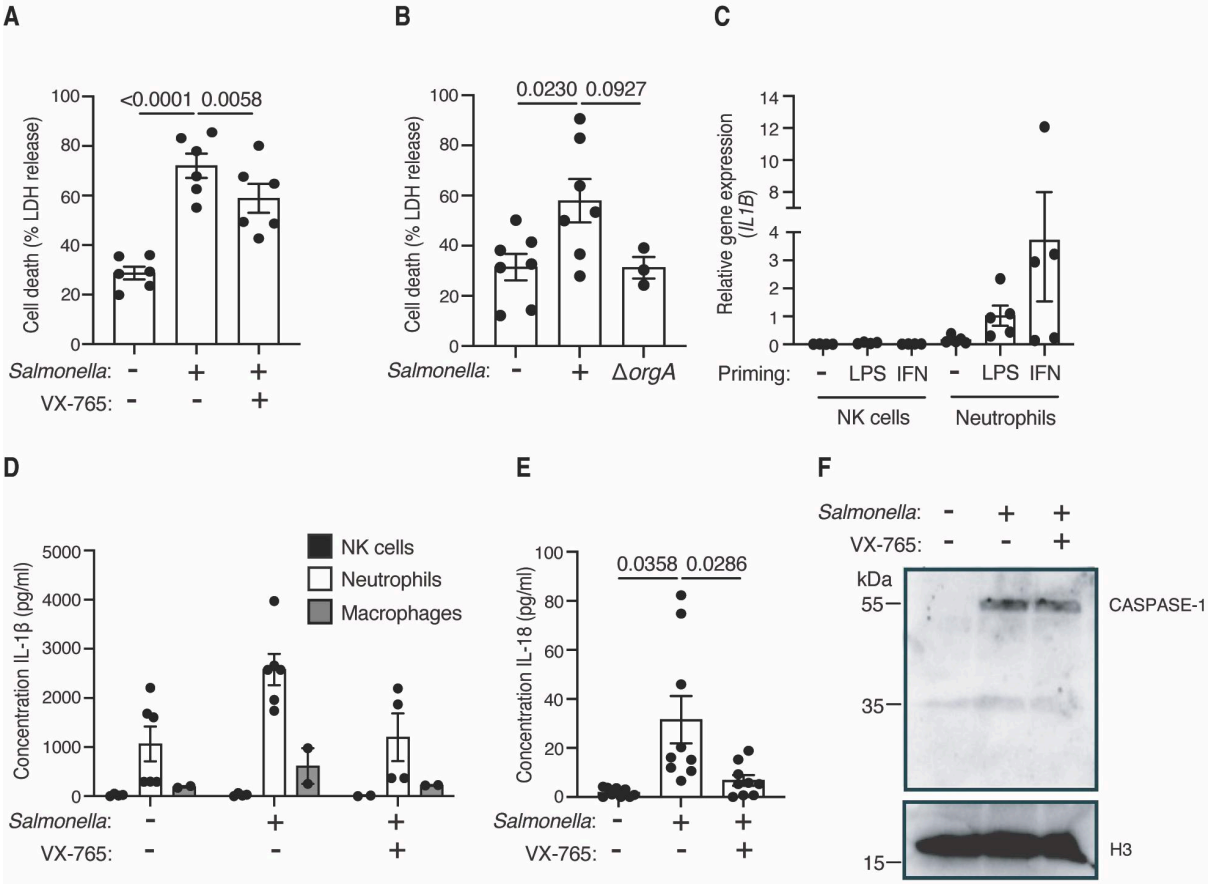

A
<0.0001
0.0058
Cell death (% LDH release)
Salmonella: - + +
VX-765: - - +
B
0.0230
0.0927
Cell death (% LDH release)
Salmonella: - + ΔorgA
C
Relative gene expression (IL1B)
Priming: - LPS IFN - LPS IFN
NK cells
Neutrophils
D
NK cells
Neutrophils
Macrophages
Concentration IL-1β (pg/ml)
Salmonella: - + +
VX-765: - - +
E
0.0358
0.0286
Concentration IL-18 (pg/ml)
Salmonella: - + +
VX-765: - - +
F
Salmonella: - + +
VX-765: - - +
kDa
55
35
15
CASPASE-1
H3

Figure 3

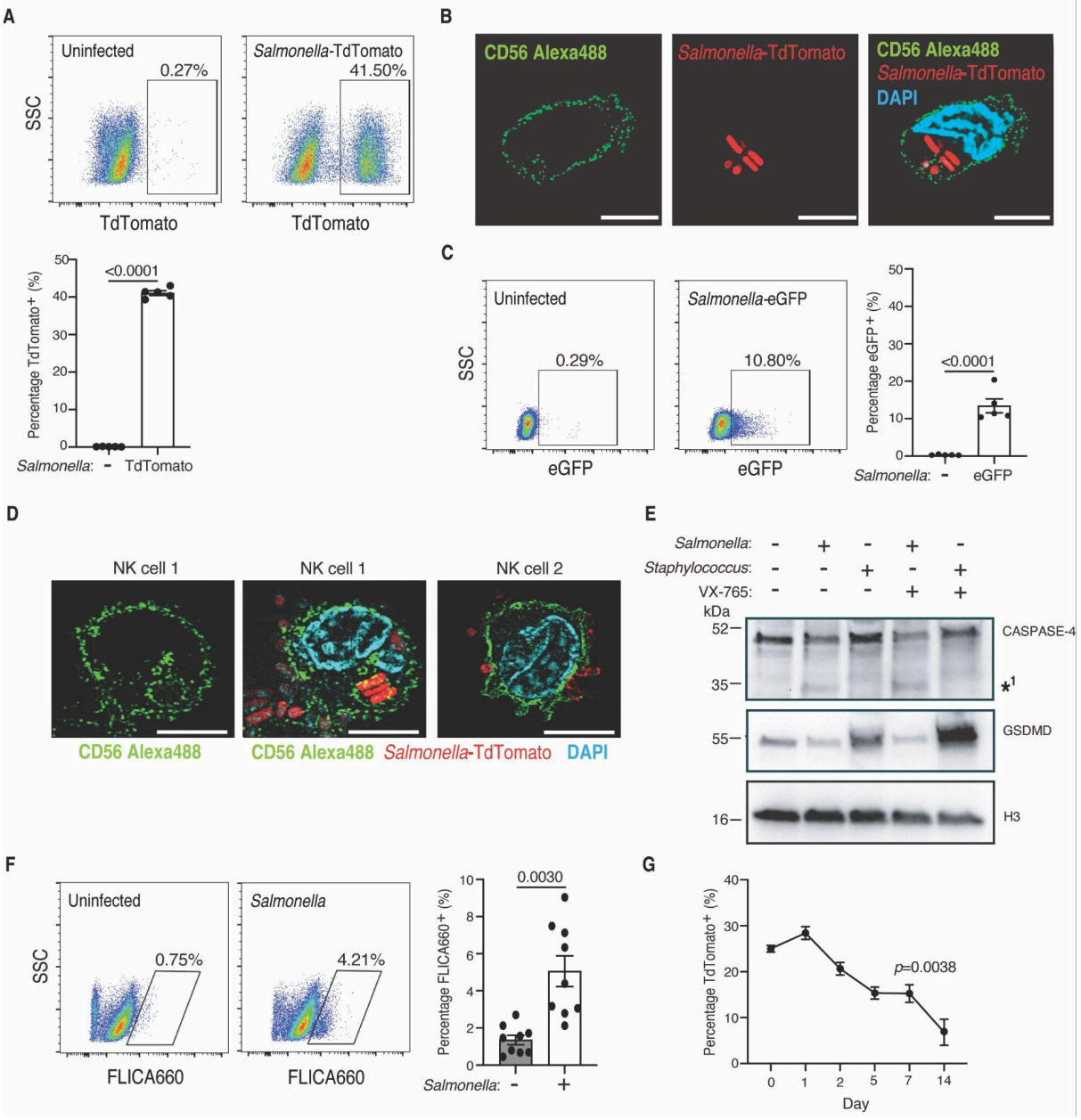
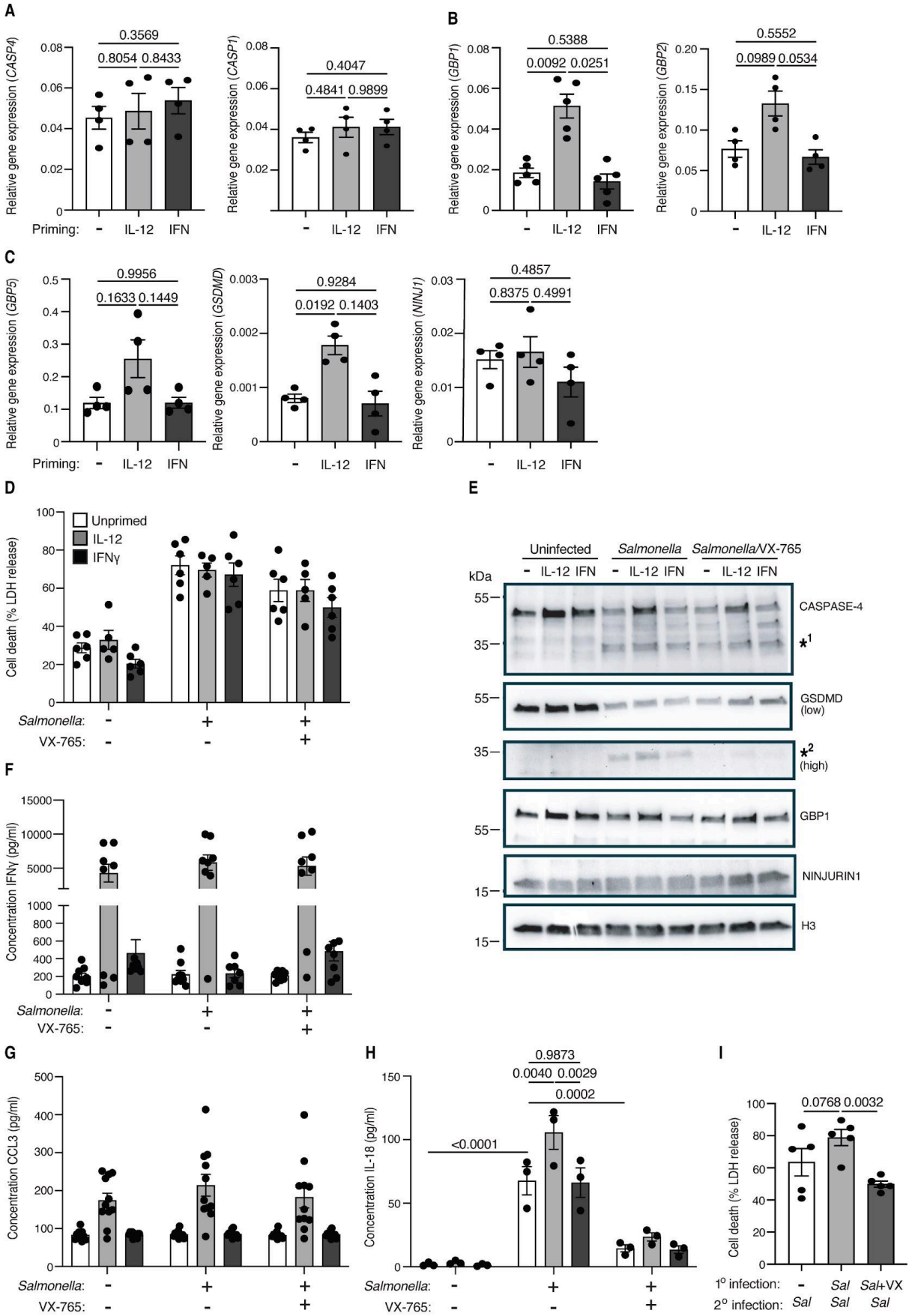

A
Uninfected
0.27%
Salmonella-TdTomato
41.50%
SSC
TdTomato
TdTomato
Percentage TdTomato+ (%)
<0.0001
Salmonella: - TdTomato
B
CD56 Alexa488
Salmonella-TdTomato
CD56 Alexa488
Salmonella-TdTomato
DAPI
C
Uninfected
0.29%
Salmonella-eGFP
10.80%
SSC
eGFP
eGFP
Percentage eGFP+ (%)
<0.0001
Salmonella: - eGFP
D
NK cell 1
NK cell 1
NK cell 2
CD56 Alexa488
CD56 Alexa488 Salmonella-TdTomato DAPI
E
Salmonella: - + - + -
Staphylococcus: - - + - +
VX-765: - - - + +
kDa
52
35
CASPASE-4
*1
55
GSDMD
16
H3
F
Uninfected
0.75%
Salmonella
4.21%
SSC
FLICA660
FLICA660
Percentage FLICA660+ (%)
0.0030
Salmonella: - +
G
Percentage TdTomato+ (%)
p=0.0038
Day

Figure 4


A
Relative gene expression (CASP4)
0.3569
0.8054 0.8433
Relative gene expression (CASP1)
0.4047
0.4841 0.9899
Priming: - IL-12 IFN
B
Relative gene expression (GBP1)
0.5388
0.0092 0.0251
Relative gene expression (GBP2)
0.5552
0.0989 0.0534
C
Relative gene expression (GBP5)
0.9956
0.1633 0.1449
Relative gene expression (GSDMD)
0.9284
0.0192 0.1403
Relative gene expression (NINJ1)
0.4857
0.8375 0.4991
D
Unprimed
IL-12
IFNγ
Cell death (% LDH release)
Salmonella:
VX-765:
E
Uninfected
Salmonella
Salmonella/VX-765
kDa
CASPASE-4
*1
GSDMD (low)
*2 (high)
GBP1
NINJURIN1
H3
F
Concentration IFNγ (pg/ml)
G
Concentration CCL3 (pg/ml)
H
Concentration IL-18 (pg/ml)
0.9873
0.0040 0.0029
0.0002
<0.0001
I
Cell death (% LDH release)
0.0768 0.0032
1° infection: - Sal Sal+VX
2° infection: Sal Sal Sal

Supplementary Table 1

A

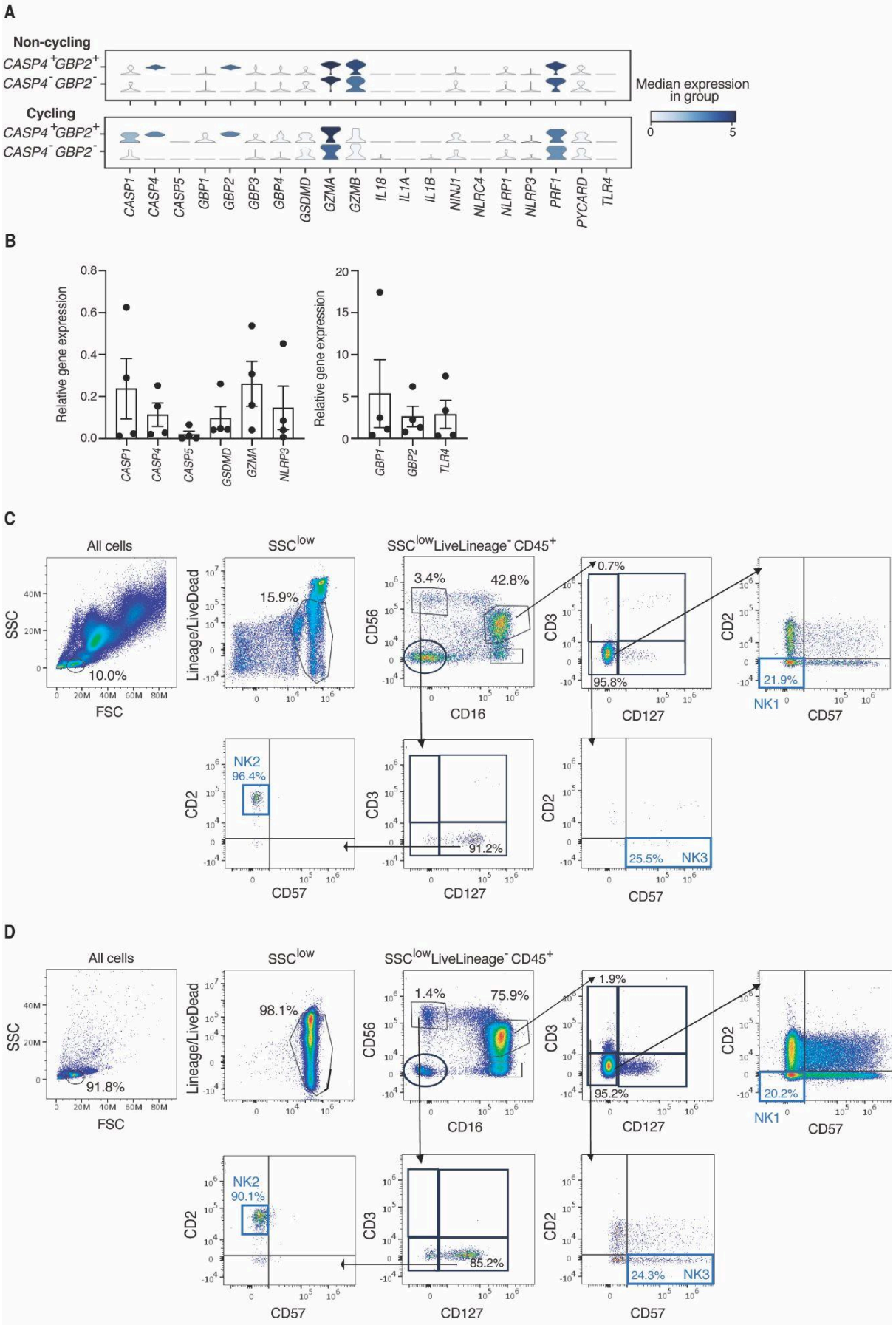
Human gene	Taqman ID
NLRP3	Hs00918062
NLRC4	Hs00892666
NINJ1	Hs00982607
TLR4	Hs00152939
GSDMD	Hs00226875
Casp1	Hs003354836
Casp4	Hs01031951
Casp5	Hs07290189
GBP1	Hs00266717
GBP2	Hs00894837
GBP5	Hs00369472
GzmA	Hs009869184
IFNGR1	Hs00166223
GAPDH	Hs02758991
IL1B	Hs01555410

Mouse gene	Taqman ID
Ninj1	Mm00479014
Gsdmd	Mm00509958
Il1a	Mm00439620
Il1b	Mm00434228
Il118	Mm00434225
Gbp2b	Mm00657086
Gbp5	Mm00463735
Ifngr1	Mm00599890
Cd14	Mm00438094
Tlr4	Mm00445273
Casp1	Mm00438023
Casp4	Mm00432304
Pycard	Mm00445747

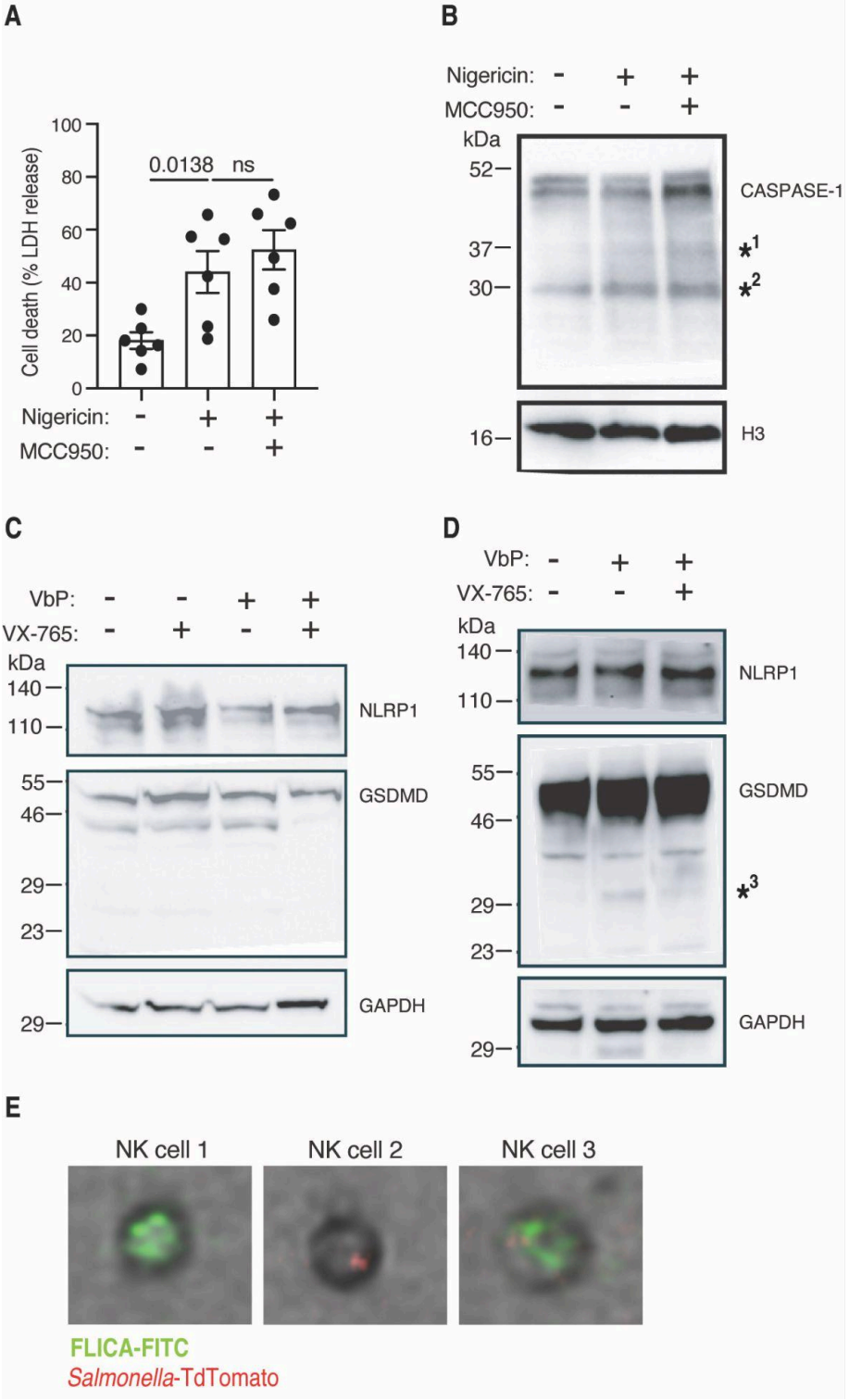
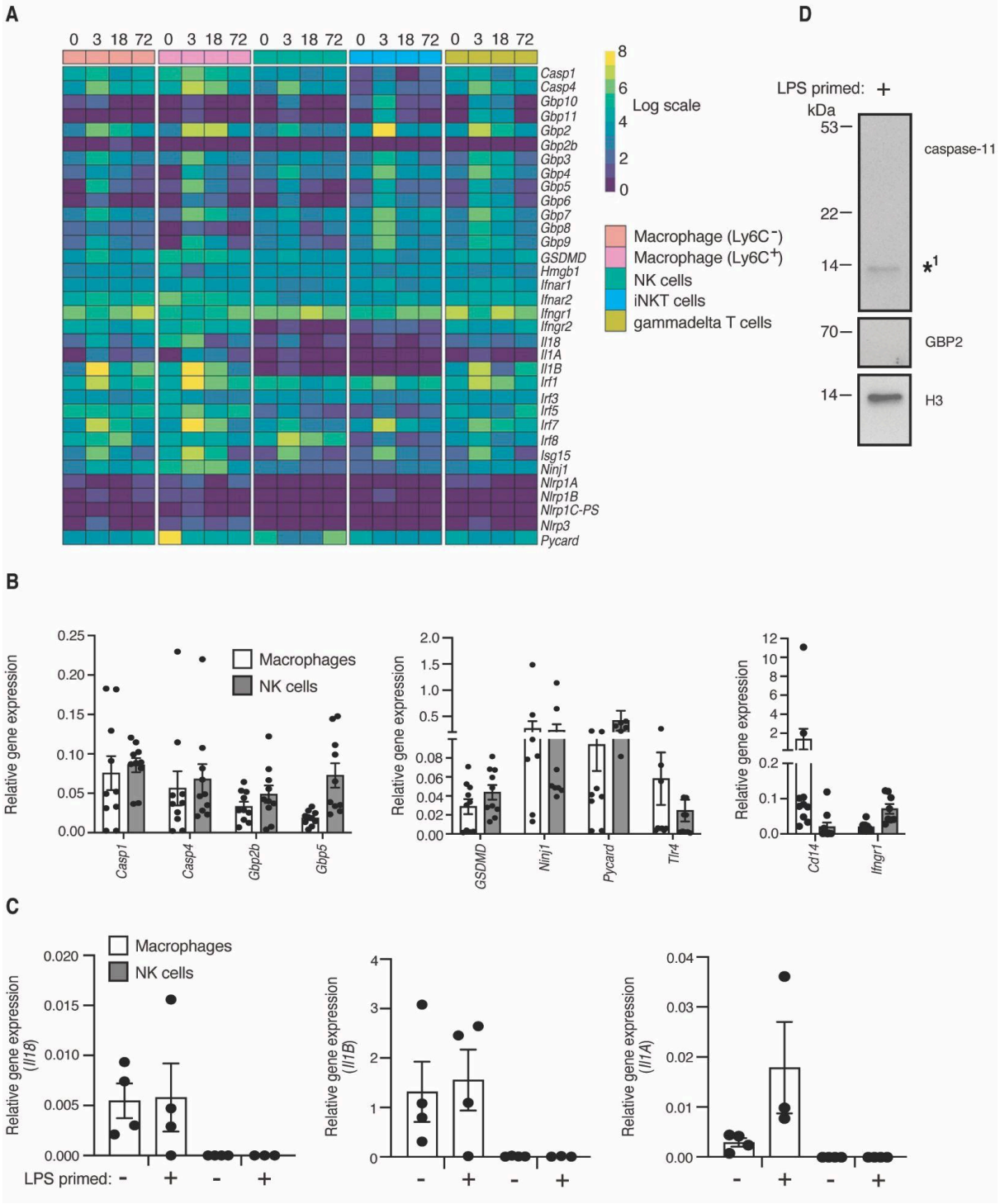
B

Human antibody target	Clone number	Catalog number	Supplier	Use
CASPASE-1	D7F10	3866	Cell Signalling	WB
GBP1	polyclonal	15303-1-AP	Proteintech	WB
GBP2	polyclonal	11854-1-AP	Proteintech	WB
GBP5	polyclonal	13220-1-AP	Proteintech	WB
αTubulin	1E4C11	66031-1-Ig	Proteintech	WB
CASPASE-4	1D1E12	67398-1-Ig	Proteintech	WB
GAPDH	1E6D9	60004-1-Ig	Proteintech	WB
NINJURIN 1	2G5	H00004814-MO1A	Abnova	WB
GSDMD	E9S1X	39754S	Cell Signalling	WB
NLRP1	9F9B12	679802	Biolegend	WB
H3	1A2A3	68345-1-Ig	Proteintech	WB
CD45 BUV395	HI30	363-0459-42	Thermo	FC
CD4 PE	SK3	60122PE	StemCell Technologies	FC
CD8a PE	HIT8a	300908	Biolegend	FC
CD19 PE	HIB19	302208	Biolegend	FC
CD14 PE	HCD14	325605	Biolegend	FC
CD123 PE	6H6	306006	Biolegend	FC
CD303 PE	201A	354204	Biolegend	FC
CD3e BV605	UCHT1	300459	Biolegend	FC
CD16 BV480	eBioCB16	414-0168-41	Thermo	FC
CD56 BUV661	TULY56	376-0566-42	Thermo	FC
CD127 PECy7	R34.34	25-1271-82	Thermo	FC
CD2 AlexaFluor700	RPA-2.10	300238	Biolegend	FC
CD57 BV421	QA17.A04	393326	Biolegend	FC
NCAM1 (CD56)	E7X9M	99746	Cell Signalling	IF
Mouse antibody target	**Clone Number**	**Catalog Number**	**Supplier**	**Use**
Caspase-11	17D9	NB120-10454	Novus Biologicals	WB
GBP2	polyclonal	11854-1-AP	Proteintech	WB
H3	1A2A3	68345-1-Ig	Proteintech	WB
CD45 AlexaFluor700	30-F11	103128	Biolegend	FC
CD3 PE	17A2	100206	Biolegend	FC
CD4 PE-Cy7	GK1.5	100421	Biolegend	FC
CD8a PE-Cy7	53-6.7	100721	BioLegend	FC
CD19 PE-Cy7	1D3	25-0193-82	eBioscience	FC
CD11b BV510	M1/70	101263	Biolegend	FC
CD11c PE-Cy7	N418	117317	BioLegend	FC
FceR1a PE-Cy7	MAR-1	134317	BioLegend	FC
Gr1 PE-Cy7	RB6-8C5	108416	BioLegend	FC
NK1.1 BV421	PK136	108732	Biolegend	FC
Other antibodies	**Clone Number**	**Catalog Number**	**Supplier**	**Use**
Mouse Ig-HRP	Polyclonal	RGAM001	Proteintech	WB
Rabbit Ig-HRP	Polyclonal	RGAR001	Proteintech	WB
Anti-rabbit IgG (H+L), (Alexa Fluor® 488 Conjugate)	F(ab')2 Fragment	4412	Cell Signalling	IF

Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3